New Methods for Detection of Low Levels of DNA Damage in Human Populations

by William A. Haseltine,*†‡ William Franklin‡ and Judith A. Lippke‡

The use of a postlabeling method to characterize and to detect infrequent base modifications in DNA is outlined. This method has the advantage that low levels of DNA modifications, approximately 1 modified base per 10^5 nucleotides, can be detected. Moreover, a broad spectrum of modification can be identified by using this methodology. The basis for the method involves transfer of a radioactive phosphate from the γ position of ATP to the 5'-hydroxyl terminus of 3'-phosphoryl nucleotides that are derived from modified DNA by appropriate nuclease digestion. The second method involves use of a defined DNA sequence within human cells. The α sequence is used as a probe for DNA damage to specific nucleotides. The α DNA sequence is reiterated approximately 300,000 times in the human genome and exists in tandem arrays. It comprises approximately 1% of the entire genome. The reiterated sequence is sufficiently homogeneous to permit its use as a probe for a site specific in DNA damage.

Examples of the application of both of these methodologies to DNA damage inflicted in human cells by chemicals and ultraviolet light are provided.

Introduction

Exposure of humans to toxic substances poses an increasing health risk throughout the industrialized world. Measurement of the health risk associated with such exposure is bedeviled by a myriad of problems. Problems begin with the accurate assessment of exposure. Exposures often occur over long periods of time to low levels of complex mixtures of chemical substances. Compounding the complexity of the problem is the likelihood that individuals respond differently to different sets of substances. Differences in individual health and metabolism may dramatically alter the affect of a given exposure.

What tools must be developed to grapple with this intractable problem? Accurate means for individual dosimetry are needed. Methods for assessment of biological damage to individuals are necessary. Means for determination of which chemical, among a variety of chemical exposures, leads to observable biological changes must be developed. Over the past few years, we have developed several new techniques that meet some of these needs. The methods were designed to provide insights into mechanisms of DNA damage and repair. Applied to the area of human toxicology, they should provide sensitive, analytical tools for determination of individual loads of DNA damage.

Postlabeling Methods to Detect and Characterize Infrequent Base Modifications in DNA

We sought to develop a method for the detection of DNA lesions that could measure low levels of damage in DNA of exposed individuals. The postlabeling method described below has the following attractive features for such work.

*Department of Pathology, Harvard Medical School, Boston, MA 02115.

†Department of Microbiology, Harvard School of Public Health, Boston, MA 02115.

‡Laboratory of Biochemical Pharmacology, Sidney Farber Cancer Institute, Boston, MA 02115.

Low levels of DNA modifications can be detected. The limit of sensitivity is probably about one modified base per 10^5.

A broad spectrum of modifications can be detected. Both small and large base adducts can be detected. Alterations in sugars and in the bases can also be resolved. Intrastrand and interstrand cross-links can be detected as well.

The specific type of damage can be determined. Each DNA modification can be analyzed independently. The contribution to DNA damage of each component of a complex mixture can be identified. Consequently, the damage created by specific environments should yield a characteristic "fingerprint" of lesions. Such fingerprint analysis could permit tracing of DNA damage to particular types of toxic waste, and permit cause-effect relationships to be established.

The persistence of each type of lesion can be measured. Measurements taken over time would permit analysis of the persistence of specific lesions.

The method would provide an individual dosimeter of effective exposure. Measurement of exposure based on ambient levels of a substance in the air or water do not measure accurately individual exposure. Measurement of serum and urine levels of substances do measure individual burdens. However, the biological affects of a given level of exposure may vary considerably from person to person. Measurement of the level of DNA damage is likely to provide a better indicator of the biological effects of exposure than other methods.

The measurements can be performed on very small tissue samples. A 1- or 2-μg quantity of DNA is all that is required for complete analysis. Such amounts of DNA can be collected from routine blood samples or from microbiopsy samples.

The Method

A new method is presented that allows the detection and characterization of infrequent base modifications in DNA. The method combines the technique of *in vitro* labeling of nucleotides derived from cellular DNA with the methods of high pressure liquid chromatography (HPLC). Previous methods for the detection of modified bases in DNA have included the use of radioactive drugs or modifying agents (1) and the uniform labeling of DNA by feeding cells radioactive DNA precursors. The method presented here differs from these previous methods as DNA is postlabeled after being treated with a DNA base-modifying agent.

An example of the DNA postlabeling method is demonstrated. DNA was treated *in vitro* with dimethyl sulfate (DMS), a DNA alkylating agent.

DMS reacts with purines in DNA (2), the principal adducts formed being 7-methylguanine, 3-methyladenine and 1-methyladenine (3). Following DMS treatment, the DNA was isolated, and digested to nucleotides. The nucleotides were then postlabeled and were resolved by HPLC. DMS-methylated bases, in nucleotide form, were readily resolved from the four normal DNA bases and were detected along with two other minor products, 5-methylcytosine and uracil.

DNA Postlabeling Procedure

The basic procedure for postlabeling DNA is presented in Figure 1. DNA is modified with a DNA damaging agent that produces stable base modifications. The modified DNA is isolated and is digested by two enzymes, micrococcal nuclease and spleen phosphodiesterase, to form 3'-deoxynucleoside monophosphates (4). The 3'-nucleotides are then labeled with ^{32}P at the 5' position by the addition of high specific activity γ^{32}P ATP and T4 polynucleotide kinase (5). A mutant enzyme that lacks the endogenous 3'-phosphatase activity (6) is used to label the
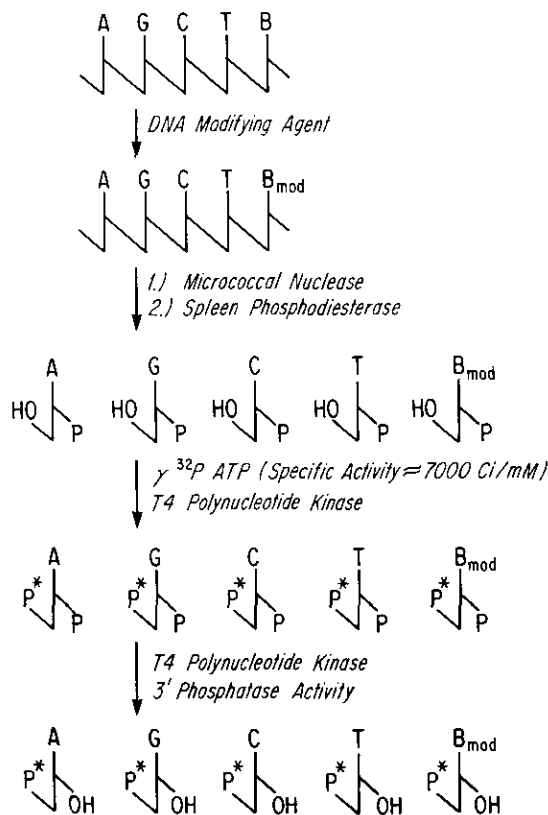

FIGURE 1.   Outline of the DNA postlabeling method.

nucleotides, the products produced being 3'5'-deoxy-nucleoside diphosphates. The 3'-phosphate group is then removed by the addition of normal T4 poly-nucleotide kinase which contains 3'-phosphatase activity (7). The normal four DNA nucleotides are labeled, as are any nucleotides containing modified bases.

Materials and Methods

Reagents and Buffers

Used were DMS, 1.22 g/L (Aldrich); $3M$ Na acetate, pH 5.5; ethanol; $0.1M$ $CaCl_2$; $0.1M$ Tris-Cl, pH 7.5; $0.05M$ Tris-Cl, pH 7.5; $0.1M$ HCl. The post-labeling reaction buffer was 16 mM $MgCl_2$. Also used were $8mM$ dithiothreitol (DTT), $50mM$ Tris-Cl, pH 7.4, 200 μg/mL bovine serum albumin (Sigma) and $\gamma^{32}P$ 5'-ATP (New England Nuclear, 1.7 μM, 3.94 mCi/mL, 3100 Ci/mmole). The deoxynucleotides —5'-dCMP, 5'-dUMP, 5'-methyl-5'-dCMP, 5'-dTMP, 5'-dGMP and 5'-dAMP (Sigma)—were used at concentrations of 0.5 mg/mL of each in $50mM$ Tris-Cl, pH 7.4.

DNA

DNA was extracted from a human lymphoblastoid cell line, TK6 (8), using a standard phenol/chloroform–isoamyl alcohol method as previously described (9). The DNA was freed of RNA by treatment with RNAse A. DNA at a concentration of 450 μg/mL was stored in $10mM$ Tris-Cl, pH 7.4, $1mM$ EDTA at 4°C.

Enzymes

DNAse I was used at 50 mg/mL (Sigma, 640 unitsmg) in $10mM$ Tris-Cl, pH 8.5, and $10mM$ $CaCl_2$. It was stored at 4°C.

Snake venom phosphodiesterase, 100 units/mL (Worthington) in $10mM$ Tris-Cl, pH 10.4, and $50mM$ $CaCl_2$, micrococcal nuclease, 100 units/μL (Worthington)and spleen phosphodiesterase, 32 units/mL (Sigma) were also stored at 4°C. T4 polynucleotide kinase (no 3'-phosphatase activity), 6000 units/mL (New England Nuclear) in $10mM$ Tris-Cl, pH 7.5, $1mM$ DTT, $50mM$ KCl, $0.1mM$ EDTA, $0.1μM$ ATP and 50% glycerol (v/v) was purified from T_4PseT_{1am} E10-infected *E. coli* (6). It was stored at -20°C. T4 polynucleotide kinase (containing 3'-phosphatase activity) was used at 1500 units/mL (New England Biolabs) in $50mM$ KCl, $10mM$ Tris-Cl, pH 7.4, $0.1mM$ EDTA, $1mM$ DTT, 200 μg/mL BSA, 50% glycerol (v/v). It was stored at -20°C.

Reaction of DMS with DNA

DNA was made up at a concentration of 400 μg/mL in $100mM$ Tris-Cl, pH 7.5. DMS (20 μL) was reacted with 180 μL of DNA for 1.5 hr at 37°C. As a control, 20 μL of $100mM$ Tris-Cl, pH 7.5, was added to 180 μL of DNA and was also incubated at 37°C for 1.5 hr. Following the incubation, 22 μL of $3M$ Na acetate, pH 5.5, and 450 μL of ethanol were added to the incubation mixture to precipitate the DNA. The tube was well mixed and placed in a dry ice/ethanol bath at -70°C for 10 min and was then centrifuged at 12000g for 10 min. The supernatant was removed and the DNA pellet was washed with 200 μL of ethanol and centrifuged at 12000g for 5 min. The supernatant was removed and the DNA was lyophilized.

DNA Digestions

5'-Digestion. Lyophilized DNA (72 μg) was reacted with 40 μL of DNAse I and 20 μL of snake venom phosphodiesterase. The DNA was incubated with the enzyme mixture overnight at 37°C.

3'-Digestion. Lyophilized DNA (72 μg) was incubated with 2 μL of $0.1M$ $CaCl_2$, 5μL of micrococcal nuclease, 20 μL of spleen phosphodiesterase, and 33 μL of 50 mM Tris-Cl, pH 7.4. The DNA was digested for 1 hr at 37°C.

5'-Labeling of 3'-Deoxynucleotides

The mixture of 3'-digested DNA was diluted 1:500 to an approximate concentration of 10μM 3'deoxynucleotides. The 3'-deoxynucleotides (5 μL = 50 pmole) were reacted with 2 μL of T4 polynucleotide kinase (New England Nuclear, no 3' phosphatase activity), 20 μL of $\gamma^{32}P$ ATP, and 49 μL of the postlabeling reaction buffer at 37°C for 2 hr. Subsequently, 4 μL of $0.1M$ HCl was added to adjust the pH to 6.0 and 10 μL of T4 polynucleotide kinase (New England Biolabs, containing 3'-phosphatase activity) was added, and the reaction was incubated for an additional 2 hr at 37°C.

In order to remove any unreacted $\gamma^{32}P$ ATP, the labeled nucleotides were separated from ATP by the use of a Bond-Elut mini-extraction column containing 100 mg of an aminopropyl sorbent (Analytichem. International, Harbor City, CA). The column packing material consists of aminopropyl moieties bonded to silica. The reaction mixture of 5'-labeled nucleotides (90 μL) was added to the top of the column, along with 500 μL of water. The column, which contains the sorbent in a 1-cm³ syringe, was placed in a 15 mL centrifuge tube and was centrifuged for 3 min at 5000 rpm in a clinical

centrifuge. The elutant was discarded, and 500 μL of 0.1M KH_2PO_4, pH 4.5, was added to the top of the column to elute the 5'-labeled nucleotides. The column was again placed in a 15 mL centrifuge tube and was centrifuged at 5000 rpm for 3 min. The elutant was collected and lyophilized. The radioactive 5'-nucleotides were resuspended in 200 μL of water.

HPLC Instrumentation and Separation Procedures

HPLC instrumentation included a Varian 5020 liquid chromatograph equipped with a Valco loop injector fitted with a 50 μL sample loop. Additional equipment included a Varian UV-50 variable wavelength detector and an ISCO model 328 fraction collector equipped with a stop-flow valve.

Separation of 5'-deoxynucleotides was performed on a Waters Bondapak C_{18} reverse phase column (300 × 4mm). A gradient elution was utilized in which an initial elution buffer concentration of 100% 75mM KH_2PO_4, pH 4.5, was maintained for 15 min, followed by a linear gradient for 15 min to a final elution mixture of 40% methanol, 60% 75mM KH_2PO_4, pH 4.5. The flow rate was maintained at 1 mL/min. UV detection of 5'-deoxynucleotides was at a wavelength of 260 nm.

For radioactivity measurements, fractions were obtained of liquid eluting from the column at intervals of 0.3 min each. Fractions were collected in 1.5 mL micro test tubes (Eppendorff). The ^{32}P radioactivity in each fraction was determined by Cerenekov counting using a Beckman LS8000 scintillation counter.

Example of Postlabeling Method
DMS Modification of DNA

To demonstrate the DNA postlabeling method, human DNA was treated *in vitro* with DMS to allow sufficient alkylation of purine bases. The DNA was then digested to both 3'- and 5'-deoxynucleoside monophosphates. The 3'-deoxynucleotides were then postlabeled with ^{32}P and were subjected to the 3'-phosphatase activity of T4 polynucleotide kinase, forming 5'-labeled deoxynucleotides. This step is included in the labeling procedure as there is much more selectivity in the separation of nucleoside monophosphates than nucleoside diphosphates by HPLC. The 5'-deoxynucleotides formed from the digestion of DNA by DNAse I and snake venom phosphodiesterase are used as markers for the radioactive nucleotides.

The use of reverse-phase chromatography as a

highly selective method for the separation of nucleotides is demonstrated in Figure 2. In this separation, the four common deoxynucleotides 5'-dCMP, 5'-dTMP, 5'-dGMP and 5'-dAMP are readily separated and resolved, as are two nucleotides containing bases less frequently found in DNA—5'-dUMP and 5'-methyl-5'-dCMP. The occurrence of 5'-dUMP is found in DNA as a result of the deamination of cytosine. The occurrence of 5-methylcytosine results from methylation of cytosine and represents about 0.4% of the total bases in DNA of HeLa cells (10). The separation of 5'-deoxynucleotides from digested human DNA is shown in Figure 3.

The separation of the postlabeled nucleotides from untreated human DNA is shown in Figure 4. The composition of each nucleotide is shown in Table 1.
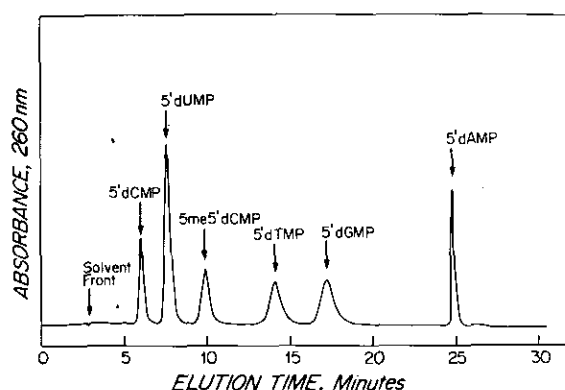

FIGURE 2. HPLC separation of 5'-deoxynucleotide: 10 μL each of 0.5 mg/mL 5'-dCMP, 5'-dUMP, 5-methyl 5'-dCMP, 5'-dTMP, 5'-dGMP and 5'-dAMP were injected on the reverse-phase column. Detection by ultraviolet absorbance at 260 nm.
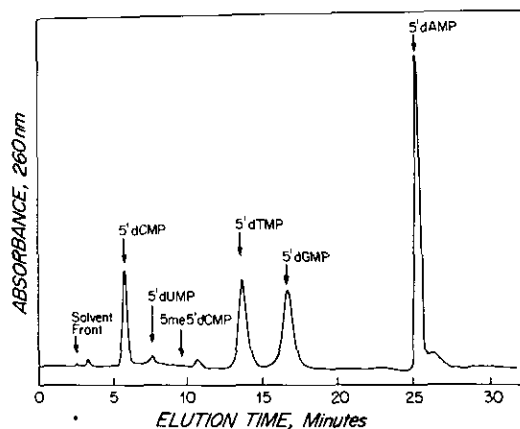

FIGURE 3. HPLC reverse-phase separation of 5'-deoxynucleotides from human DNA digested with DNAse I and snake venom phosphodiesterase. Detection by ultraviolet absorbance at 260 nm.

Table 1. Percentages of bases for control DNA and DNA treated with DMS.

Base	Content of bases, %	
	Control (no treatment)	DMS treatment
5'-dCMP	23.8	29.5
5'-dUMP	1.07	1.70
5'-Me-5'-dCMP	0.57	0.83
5'-dTMP	24.2	28.7
5'-dGMP	22.8	5.52
5'-dDMP	27.0	15.7
DMS products	—	18.1

It is noted that the content of 5-methylcytosine is 0.6%, which is comparable to a published value of 0.4% for human DNA (*10*).

The treatment of human DNA with DMS resulted in the formation of modified bases, as is evident in Figure 5. Three new eluting products are seen in the HPLC separation of the labeled 5'-deoxynucleotides. The three new peaks most likely result from purine methylation, as the percentage of normal purine bases is less than in the untreated DNA (see Table 1). The identification of each DMS product was not possible, as markers were not available for 5'-deoxynucleotides containing methylated purines. However, preliminary evidence from the treatment of 5'-dGMP and 5'-dAMP with DMS indicates that the product which elutes prior to 5'-dTMP results from the methylation of adenine, as does the product which elutes immediately after 5'-dAMP (data not shown). The product eluting most distant from the solvent front may be formed from the methylation of guanine.

Discussion

A new method has been presented allowing the detection of infrequent base modifications in DNA without the use of either radioactive DNA precursors or radiolabeled drugs or mutagens. Given the high specific activity of γ^{32}P-ATP available and the high resolution of HPLC reverse-phase chromatography, infrequent base modifications in the range of
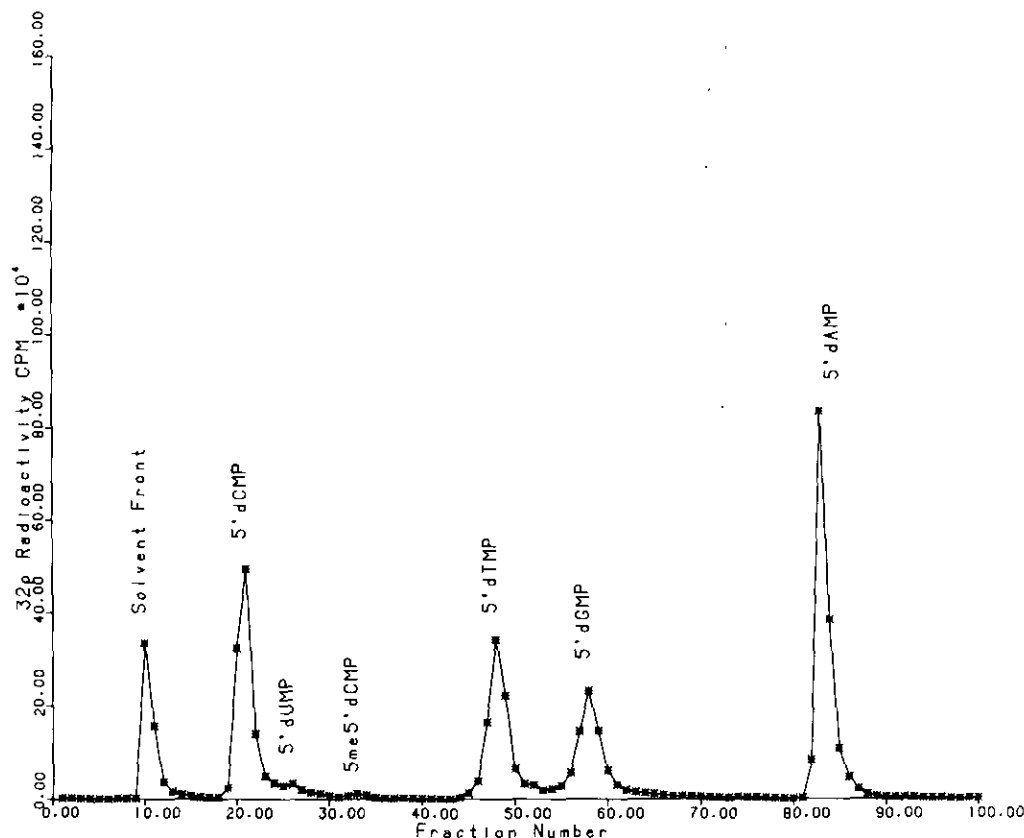

FIGURE 4. HPLC reverse-phase separation of ^{32}P-labeled 5'-deoxynucleotides from human DNA. Fractions (*) were collected every 0.3 min, and radioactivity was measured by Cerenekov counting.

one in 10^5 should be detectable. The method is also useful for the detection of modified nucleotides which can be incorporated into DNA. Once such nucleotide is cytosine arabinoside monophosphate (ara-CMP). The nucleoside cytosine arabinoside (araC) is used as an antitumor agent in the treatment of several kinds of leukemia (11). Once incorporated into DNA, ara-CMP can be isolated and has been resolved by HPLC (12). The 3'ara-CMP can be labeled by the DNA postlabeling method, and the resulting formation of 5'-ara-CMP is shown in Figure 6. It should also be possible to label other nucleotide analogs which can be incorporated into DNA.

damage within defined DNA sequences. It also has the advantage that such damage can be determined within intact human cells. Thus, cells from individuals that have been exposed to high levels of toxic substances can be examined at the level of individual nucleotides for DNA damage.

It is unlikely that the use of the alphoid sequence will be suitable for routine analysis of field samples. Limit of detection of DNA lesions using this method is about one alteration per thousand nucleotides. However, the method will serve as an important tool to determine the types of DNA damage that are created by noxious chemicals. For this analysis, the chemical composition of the damaging mix need not be known.

Use of the Alphoid Sequence as an Indicator of DNA Damage

We have also developed an analytical method for detection of DNA damage within defined sequences of human DNA. This method has the advantage that it permits determination of distribution of DNA

The Method

For this work we used the alphoid sequence of human DNA (13–16). The alphoid sequence is ideal for this purpose. It is a highly reiterated sequence present in about 300,000 copies per haploid genome. The alphoid sequence is present in tandem arrays
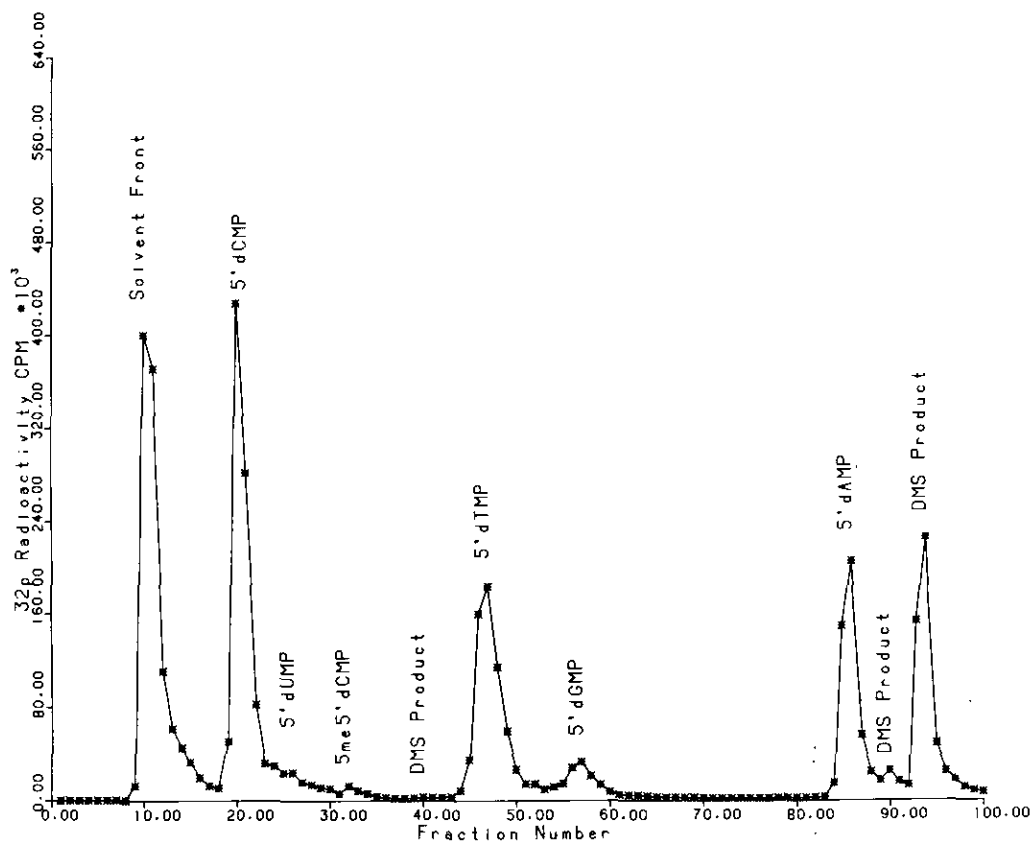

FIGURE 5. HPLC reverse-phase separation of ^{32}P-labeled 5'-deoxynucleotides from DMS-treated human DNA. Fractions (*) were collected every 0.3 min and radioactivity was measured by Cerenekov counting.
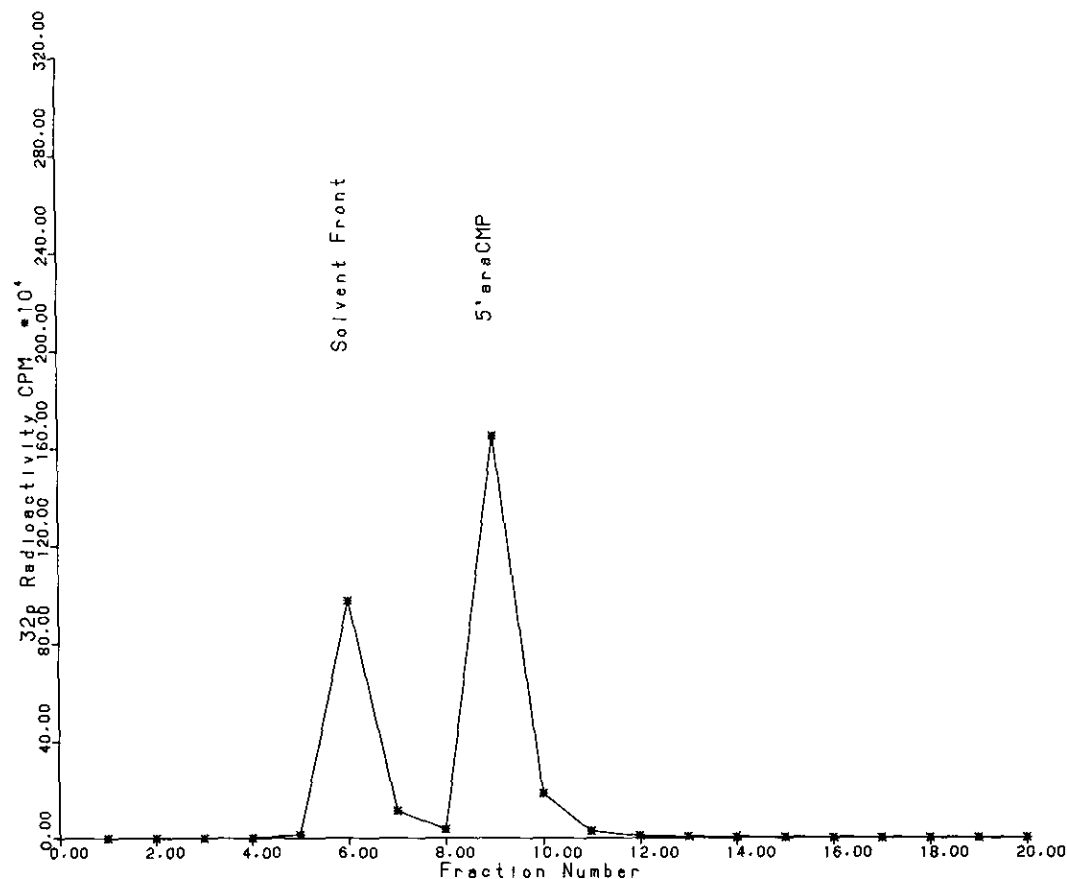
FIGURE 6. HPLC reverse-phase separation of ³²P-labeled 5'-araCMP. Fractions (*) were collected every 0.5 min and radioactivity was measured by Cerenekov counting.

up to 40 repeat units in length (15). The basic repeat length of the human alphoid sequence is 342 nucleotides (13). This sequence is itself derived from an imperfect repeat unit of 171 nucleotides. The alphoid sequence comprises about 1% of the total mass of human DNA. For a thorough treatment of the structure of the human alphoid sequences, see the article by Wu and Manuelidis (15).

The alphoid DNA can be readily obtained from total genomic extracts. The detailed methods of isolation are presented below. Figures 7 and 8 are schematic illustrations of the isolation and labeling procedure. Total DNA is prepared from intact cells. Any human tissue or cultured cell line may be used, as the alphoid sequence is present in all normal and tumor cells. After purification, total cellular DNA is treated with a restriction enzyme, Eco RI. The alphoid sequence contains an Eco RI cleavage site. Treatment with Eco RI releases a major fraction of the alphoid DNA as a fragment of 342 nucleotide length as a direct consequence of the tandem array organization of the alphoid DNA. This 342 base pair

fragment is easily separated from the bulk of the cellular DNA that is cleaved into fragments of between 3000 and 6000 base pairs (Fig. 9). For the analysis, the Eco RI alphoid fragment can be labeled at either the 3' or 5' terminus. DNA fragments that contain a single labeled terminus are obtained by treatment of the Eco RI fragment with restriction enzymes that cleave the DNA. A restriction enzyme cleavage map of the Eco RI alphoid DNA is pictured in Figure 10. We have used both the Eco RI* of 92 base pair length and the Mbo I fragments of 52 base pair length for our work.

If the human alphoid sequence is to be useful for these studies, it must have a defined sequence. Fortunately this is the case for both the Eco RI/Eco RI* and the Eco RI/Mbo I alphoid fragments. Figure 11 depicts the products of the Maxam-Gilbert sequencing reactions (17) applied to the Eco RI/Eco RI* 92 base pair fragment. It is evident that a single, unambiguous sequence can be deduced from this gel. The sequence of the Eco RI fragment as reported by Manuelidis et al. (13, 15) and confirmed
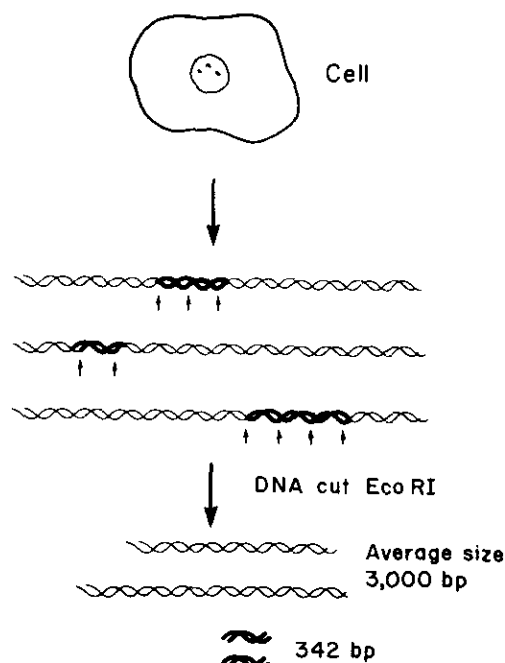
FIGURE 7. Schematic diagram of the DNA extraction procedure. Total cellular DNA is extracted from cells and then digested with the restriction endonuclease Eco RI yielding the highly reiterated 342 base pair alphoid sequence.
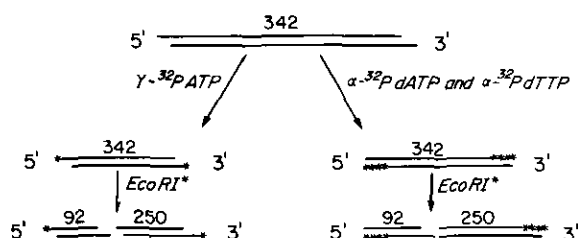

FIGURE 8. Schematic diagram of the labeling procedure and subsequent digestion with Eco RI*. The 342 base pair fragment is labeled with either $(\alpha\text{-}^{32}P)$ dATP and $(\alpha\text{-}^{32}P)$dTTP or with $(\gamma\text{-}^{32}P)$dATP and then cut with restriction endonuclease Eco RI*, yielding a 92 base pair fragment labeled at one terminus.

FIGURE 9. Human DNA digested with the restriction endonuclease Eco RI run on a 5% acrylamide gel stained with ethidium bromide. This gel illustrates the highly reiterated 342 and 680 base pair tandem repeats.

a number of cell lines including the HeLa cells, the TK6 line of human B lymphoblasts (8) and the CEM human lymphoblastic leukemia cell line (19). We have also used normal and transformed human fibroblasts grown in cell culture.

DNA Purification

The first step of the procedure involves purification of the cellular DNA. For this work the starting cells should be washed free of culture medium. Then 1×10^6 cells/mL in 100 mL grown in suspension are pelleted and washed in phosphate buffered saline. The pellet is resuspended in SSC ($0.15M$ NaCl/0.015 sodium citrate). Sodium dodecyl sulfate (SDS) is added to a final concentration of 1%. Proteinase K (2.5 mg/mL, predigested for 30 min at 37°C) is added to a concentration of 0.01 mg/mL and is incubated for 30 min at 50°C. Proteinase K is

in part by our work is shown in Figures 10 and 11.

The alphoid sequence of human DNA can be used as an indicator to compare DNA damage inflicted *in vitro* to damage created upon treatment of the same agent *in vivo*. We have used this procedure to study DNA damage in both situations created either by ultraviolet light (18) or by nitrogen mustard (9). The example of ultraviolet light damage taken from our previous work (18) is given below. Any human tissue or human cell in culture can be used for preparation of the alphoid sequence. We have used
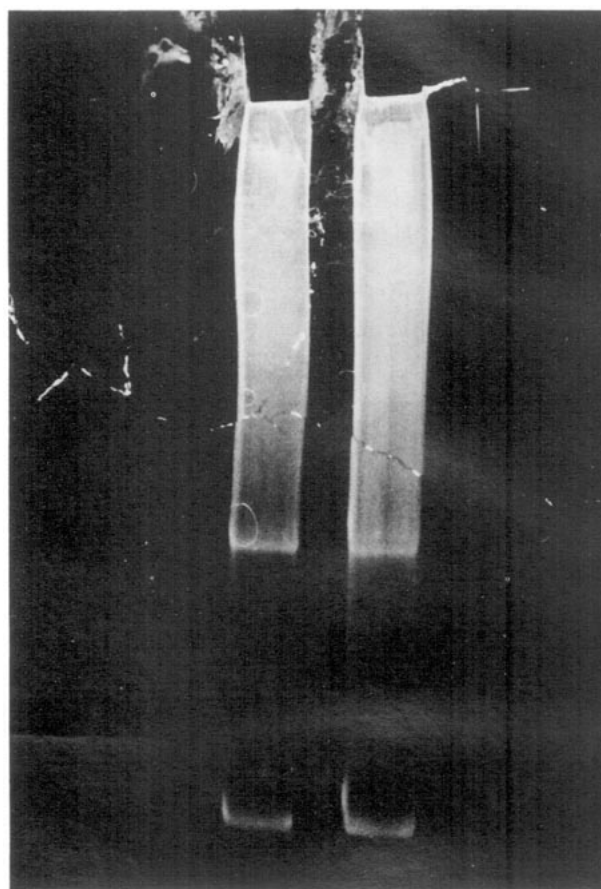
	20	30	40	50
DdeI				
AATTCTCAFG	AACTTCCTTG	ATTCAACTCA	CAGAGTTGAA	
TTAAGAGTCA	TTGAAGGAAC	ACAACACACA	TAAGTTGAGT	GTCTCAACTT

MboI 60	70	80	90	EcoRi*100
CGATCCTTTA	CACAGAGCAG	ACTTGAAACA	CTCTTTTTGT	GGAATTTGCA
GCTAGGAAAT	GTGTCTCGTC	TGAACTTTGT	GAGAAAAACA	CCTTAAACGT

110	Fnu4HI	130	140	MboII
AGTGGAGATT	TCAGCCGCTT	TGAGGTCAAT	GGTAGAATAG	GAAATATCTT
TCACCTCTAA	AGTCGGCGAA	ACTCCAGTTA	CCATCTTATC	CTTTATAGAA

160	170	HinFI
CCTATAGAAA	CTAGACAGAA	TGATTC
GGATATCTTT	GATCTGTCTT	ACTAAG

FIGURE 10. The 171 base pair sequence of human alphoid DNA. The restriction endonucleases which digest this sequence are listed above the recognition sequence.
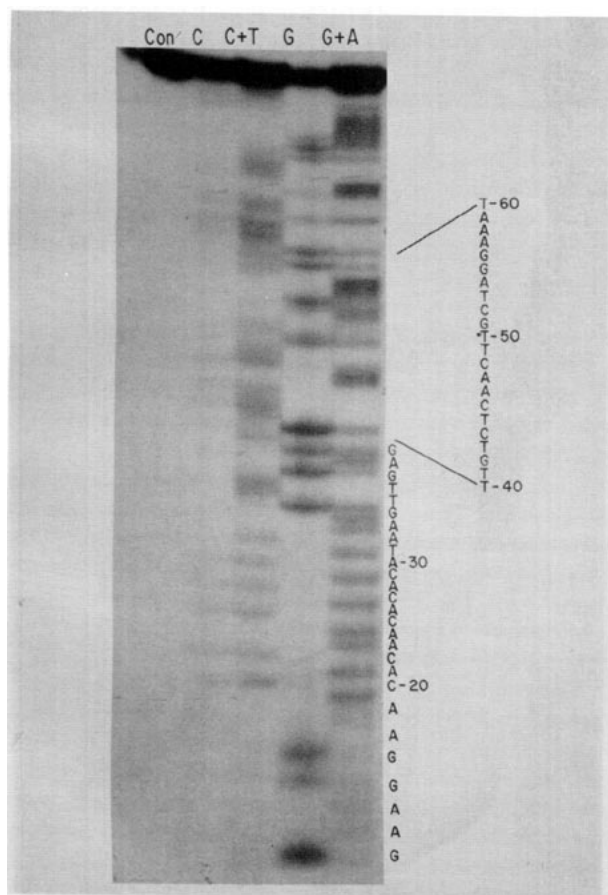

FIGURE 11. Products of the Maxam and Gilbert sequencing reactions. The 92 base pair 3′ α^{32}P-labeled alphoid sequence is treated for sequence analysis: (lane 1) control, no treatment; (lane 2) treated with hydrazine in the presence of NaCl for analysis of C; (lane 3) treated with hydrazine for analysis of C + T; (lane 4) treated with DMS (dimethyl sulfate) for analysis of G; (lane 5) treated with piperidine formate for analysis of G + A.

added again to the same concentration and incubated for an additional 30 min at 50°C. This mixture is then extracted two times with phenol (redistilled) that has been saturated with SSC and extracted two additional times with a mixture of chlorophorm and isoamyl alcohol (19:1, vol/vol). The nucleic acids are precipitated by addition of two volumes of cold 95% ethanol. The DNA is collected by spooling on a glass rod. The precipitate is resuspended in 2 mL of SSC, and ribonuclease A (2 mg/mL) is added to a final concentration of 0.04 mg/mL. The mixture is incubated at 37°C for 30 min. Proteinase K (2.5 mg/mL) is added to a concentration of 0.025 mg/mL and incubated at 37°C for 30 min. The mixture is then brought to a final volume of between 10 and 15 mL with SSC and extracted two times with phenol and two additional times with the chlorophorm isoamyl alcohol mix indicated above. The DNA is precipitated with 2 volumes of 95% ethanol and spooled. The DNA is then lyophilized.

Eco RI Restriction Enzyme Cleavage of Human DNA.

The lyophilized DNA is resuspended in the Eco RI cleavage buffer (100mM Tris-HCl, pH 7.5; 50mM NaCl; 5mM MgCl$_2$; 100 μg/mL bovine serum albumin) and is digested with the restriction enzyme, Eco RI, at a concentration of 3 units of enzyme to 1 μg of DNA at 37°C for 3 hr. Sodium ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 25mM to terminate the reaction.

To isolate the alphoid DNA from the bulk of the cellular DNA, the Eco RI digest is centrifuged through a 4.6 mL 15–30% sucrose gradient at 50,000 rpm in a Beckman VTi-65 rotor for 90 min at 20°C.

The buffer for the sucrose gradient is 1X Agarose buffer (see below). The gradient is fractionated, and 20 μL of each fraction is analyzed on a 2% agarose gel containing ethidium bromide (0.003 mg/mL). The agarose gel contains 3 g of agarose in 150 mL. The gel buffer is $0.04M$ Tris-HCl, pH 8.0; $0.018\ M$ NaCl, $0.02M$ EDTA. The fractions containing the 342 base pair alphoid sequence are located by visualization of the ethidium bromide stained bands using shortwave ultraviolet light. The gel is photographed with a Polaroid MP-3 industrial camera and type 57 (4 × 5) Land film. There should be a discrete band visible in the fractions containing the 342 base pair fragment. These fractions are then pooled and precipitated with ethanol. The precipitate is lyophilized and stored in $10mM$ Tris-HCl, pH 8.0, $1mM$ EDTA at -20°C. An alternative to visualization of the alphoid sequence using ethidium bromide stained gels is the inclusion of a labeled restriction fragment of suitable length in the sucrose gradient.

Terminal Labeling of the Alphoid 342 Base Pair Fragment

The 3'-termini can be labeled in reactions that contain the Klenow fragment of DNA polymerase (11 units), and 30 μCi each of (α-^{32}P)dATP and (α-^{32}P)dTTP, $7mM$ Tris-HCl, pH 7.5, $7mM$ MgCl$_2$, $50mM$ NaCl, $0.1mM$ DTT, $0.01mM$ dGTP, $0.01mM$ dCTP and that are incubated for 50 min at 10°C. Following this incubation, $0.01mM$ dATP and $0.01mM$ dTTP (unlabeled) are added, and the reaction mixture is incubated for an additional 10 min at 10°C (20). The DNA is then precipitated by addition of sodium acetate to a final concentration of $0.3M$, 10 μg of the tRNA and two volumes of 95% ethanol. The mixture is chilled to -70°C in a dry ice–ethanol bath for 15 min. The mixture is then centrifuged at 15,000g in an Eppendorf centrifuge for 15 min. The supernatant is discarded and the pellet is washed with 95% ethanol and then lyophilized.

For labeling of the 5' terminus the DNA is incubated in a reaction containing $100mM$ imidizole, pH 6.6, $20mM$ MgCl$_2$, $8mM$ DTT, $0.2mM$ spermine, $0.2mM$ EDTA, $0.6mM$ ADP, $16mM$ MgCl$_2$, and 1.5 units of polynucleotide kinase. The reaction also includes 250 μCi of (γ-^{32}P)dATP that has been lyophilized in the tube prior to addition of the other reaction components. The reaction mixture is incubated at 37°C for 60 min. The labeled DNA is precipitated by the addition of ammonium acetate to a final concentration of $0.3M$ and 2 volumes of 95% ethanol.

Isolation of the 92 Base Pair End-Labeled Eco RI/Eco RI* Fragment of Alphoid DNA

The alphoid DNA which is labeled at both termini is then digested with another restriction enzyme to obtain fragments that are labeled at a single terminus. To obtain a 92 base pair alphoid fragment derived by cleavage of the 342 base pair fragment with Eco RI*, the labeled DNA is resuspended in the Eco RI* reaction buffer which contains $25mM$ Tris-HCl, pH 8.6, $2mM$ MgCl$_2$, and 20% glycerol (21). The reaction is incubated for 1 hr at 37°C with 70 units of Eco RI. A mixture of two dyes, bromophenol blue and xylene cyanol, both at a concentration of 0.05%, is added to the reaction mix. This preparation is layered directly onto a polyacrylamide gel for separation of the cleaved fragments.

For isolation of the 92 base pair fragment, the cleaved DNA is layered onto a 5% polyacrylamide gel made with TEB buffer ($50mM$ Tris-HCl, $50mM$ boric acid, $1mM$ EDTA). The gel is polymerized with 0.07% ammonium persulfate and TEMED (N,N,N'N'-tetramethylethylenediamine). The gel is run at 400 V until the slow-moving dye has migrated halfway down a 40 cm long gel. In order to isolate the gel fragment, the 92 base pair sequence is located by autoradiography and the fragment excised from the gel. DNA is removed from the gel fragment by crushing the gel piece with a siliconized glass rod in a 1.5 mL Eppendorf tube. A solution of $0.3M$ sodium acetate is added to the crushed gel and the mixture is rotated overnight at room temperature. The gel mixture is spun for 3 min in an Eppendorf centrifuge, and the supernatant is removed and saved. The crushed gel is washed with $0.3M$ sodium acetate twice until most of the radioactivity has been eluted from the crushed gel. The supernatants are pooled and filtered through a 0.45 μm cellulose acetate filter. The DNA is precipitated by an addition of two volumes of 95% ethanol. The 92 base pair fragment is then ready for use.

Results

The alphoid sequence can be used to compare DNA damage created by treatment of DNA *in vitro* to damage deposited in the same DNA sequence when that sequence is part of the intact cells (Fig. 12). An experiment of this type is described using ultraviolet light as the insulting agent.

The 3' end-labeled 92 base pair Eco RI/Eco RI* alphoid sequence was prepared from cells that were either untreated or treated with 5000 J/m^2 of 254 nanometer light from a germicidal lamp. A fraction of the DNA extracted from the untreated cells was
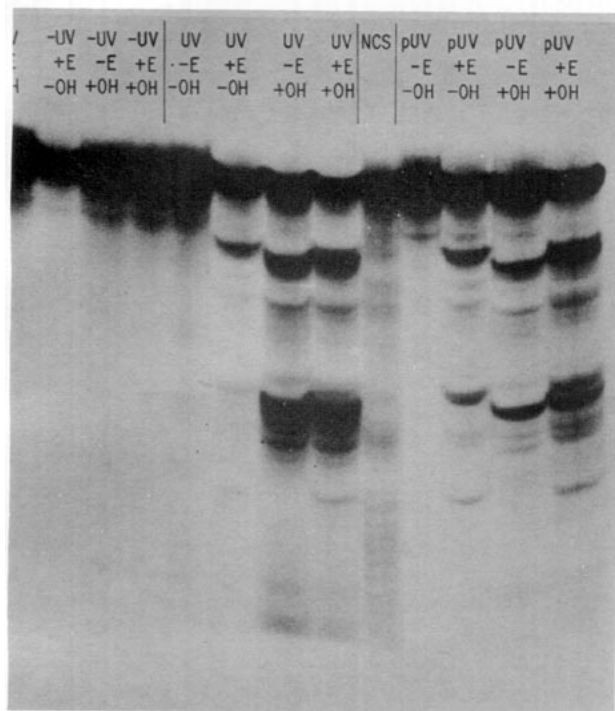
in DNA irradiated *in vitro* and within intact cells can be compared by analysis of the cleavage patterns. The relative amount of damage at each site can be determined by measurement of the amount of radioactivity in gel slices that correspond to discrete bands in the autoradiograms. The amount of damage at six specific sites in the alphoid DNA as a function of increasing ultraviolet dose is plotted in Figures 13 and 14. These data demonstrate that the sites at which DNA damage occurs are similar in
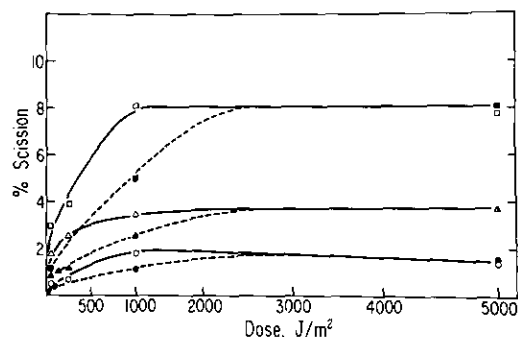
FIGURE 12. Comparison of ultraviolet-induced damage to cellular DNA and purified·DNA: (lanes 1-4) no irradiation; (lanes 5-8) irradiation of purified DNA at 5000 J/m²; (lane 9) NCS sequencing rx; (lanes 10-13) irradiation of intact cells at 5000 J/m²; (lanes 1, 5, 10) control, no treatment; (lanes 2, 6, 11) treated with *M. luteus* pyrimidine dimer endonuclease; (lanes 3, 7, 12) treated with 1*M* piperidine at 90°C for 20 min; (lanes 4, 8, 13) treated with *M. luteus* pyrimidine dimer endonuclease followed by treatment with 1*M* piperidine at 90°C for 20 min. The 92 bp fragment was prepared as described and layered on a 8% polyacrylamide urea containing sequencing gel (*17*).

FIGURE 13. Dose-response for UV light-induced damage to human alphoid DNA for (●,▲,■) intact cells and (○,△,□) purified DNA. Dose-response of pyrimidine dimer damage was determined by treatment of the irradiated DNA with the *M. luteus* pyrimidine dimer-specific endonuclease followed by resolution of the scission products on urea containing polyacrylamide sequencing gels. Scission occurred at the sequences indicated by the asterisk (○,● G-T*-T-G (37'-34'); (△,▲), G-T*-T*-C-A (51'-47'); (□,■) G-T*-T*-T*-C-A (79'-74'). The positions of the sequences within the alphoid DNA are indicated by the numbers in parentheses. The percentage of scission is the fraction of the input molecules broken at the sequences indicated.
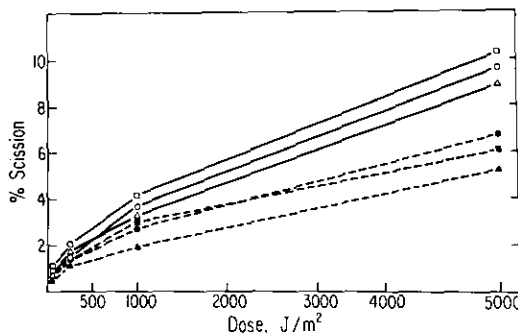
also treated with 5000 J/m² from the same source. These three samples were analyzed on a polyacrylamide gel after (1) no treatment, (2) treatment with a preparation of the ultraviolet-specific endonuclease purified from *M. luteus* that cleaves the DNA at sites of cyclobutane pyrimidine dimers or (3) treatment with 1*M* piperidine for 20 min at 90°C [a treatment that cleaves the DNA at PyC lesions (*18*)] and treatment with the *M. luteus* enzyme followed by treatment with 1*M* piperidine as before.

The results of such an experiment are illustrated in Figure 12. The alphoid DNA extracted from untreated cells is not broken by any of the treatments. Treatment of ultraviolet-irradiated alphoid DNA with piperidine or ultraviolet-specific endonuclease creates strand breaks at specific sites which are evident as DNA fragments that migrate more rapidly than the intact 92-mer. The sites of damage

FIGURE 14. Dose-response for ultraviolet light-induced damage to human alphoid DNA for (●,▲,■) intact cells and (○,△,□) purified DNA. Dose-response for alkali-labile lesions was determined by treatment of the irradiated DNA with 1*M* piperidine at 90°C for 20 min prior to layering on the gel. The percentage of scission was computed as in Fig. 13. Alkali-induced scission occurred at the sequences indicated by asterisks: (○,●) A-C-T-C*-T-G (46'-41'); (△,▲) G-T-T-C*-A (51'-47'); (□,■) G-T-T-T-C*-A (79'-74').

Table 2. Stability of the ultraviolet light-induced damage.[a]

| Treatment | UV intensity, J/m^2 | Strand scission, % | | Site[b] |
		0 hr	24 hr	
PyrDEase	100	0.66	0.49	A-C-T-C-T-G
		0.59	0.75	G-T-T-C-A
		0.77	0.73	G-T-T-T-C-A
Piperidine	100	3.79	2.60	A-C-T-C-T-G
		1.51	1.22	G-T-T-C-A
PyrDEase and piperidine	100	4.48	4.48	A-C-T-C
		3.03	2.50	G-T-T-C-A
		4.35	5.78	G-T-T-T-C-A
PyrDEase	1000	0.98	2.40	G-T-T-G
		2.35	2.70	A-C-T-C-T-G
		2.53	2.70	G-T-T-C-A
		4.60	4.43	G-T-T-T-C-A
Piperidine	1000	3.06	2.68	G-T-T-C-A
		3.42	3.29	G-T-T-T-C-A
PyrDEase and piperidine	100	7.63	6.81	G-T-T-T-C-A

[a]CEM cells were irradiated with either 100J/m^2 OR 1000 J/m^2 of UV light. DNA was extracted immediately from half of the cells and analyzed for the distribution of either pyrimidine dimers or alkaline-sensitive lesions as described in the text by treatment of the DNA with either the *M. luteus* pyrimidine dimer-specific endonuclease (PyrDEase) or with 1*M* piperidine at 90°C for 20 min or with PyrDEase followed by piperidine treatment. The remaining cells were resuspended in complete medium and incubated for 24 hr at 37°C prior to extraction of the DNA. In both cases the 3' end-labeled 92 base pair α-DNA fragment was prepared, and the amount of strand scission is shown as percentage of input molecules broken at the sequences indicated.

[b]The asterisks indicate the sites of breakage within the sequence. Sequences studied included G-T-T-G (37'-34'), A-C-T-C-T-G (46'-41'), G-T-T-C-A (51'-47'), and G-T-T-T-C-A (79'-74'), in which the numbers indicate the position on the 3' end-labeled strand relative to the labeled Eco RI end.

the two situations. However, the effective dose seen by the DNA in intact cells is about 50% of the effective dose for the same fragment when it is irradiated as naked DNA. This is the case over the entire dose range of 50–5000 J/m^2.

This method can also be used to judge the physiological stability of individual DNA lesions. Thus, the PyC and cyclobutane dimer lesions are stable in cellular DNA when the cells are exposed to very high doses, i.e., 5000 J/m^2 of ultraviolet light (Table 2). It is hoped that this method will be useful for detection of intermediates in the DNA repair pathways when lower doses of ultraviolet light are used. This method is capable of detecting damage induced by low ultraviolet fluences. We have detected lesions in alphoid DNA exposed to as little as 15 J/m^2 of ultraviolet light.

Summary

The alphoid DNA provides a convenient tool for the analysis of DNA damage within intact cells at the level of individual nucleotide sequences. It should provide a sensitive tool for the study of DNA.

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